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Screening Test for Insecticides Interfering with Cuticular Sclerotization†

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Abstract: The potential of known and new insecticides to interfere with cuticle sclerotization was investigated using assays for key enzymes such as phenoloxidase, quinone methide isomerase and DOPA decarboxylase. Homogenates from the blowfly *Lucilia cuprina* and from the epithelial cell line from *Chironomus tentans* were used to examine the compounds under investigation. Phenoloxidases are known to oxidize DOPA, the substrate for DOPA decarboxylase. Since phenoloxidases were not detectable in *C. tentans* cell homogenates, inhibitor and kinetic studies were done for comparison with DOPA decarboxylase of this insect cell line. DOPA decarboxylase and phenoloxidase of *L. cuprina* exerted highest specific activities at early pupal stages (day 7). The apparent K_m values for the two enzymes were 0·47(±0·21) mM and 0·71(±0·16) mM, respectively, using L-DOPA as substrate. DOPA decarboxylase from *C. tentans* had a K_m value of 0·42(±0·18) mM. Quinone methide isomerase was most active in young pupae. In terms of substrate specificity for enzymic (mushroom-tyrosinase) production of different quinones from their corresponding catechols, that with dopamine quinone proved to be the most efficient. Synthesis of derivatives of L-DOPA and L-tyrosine led to a compound which inhibited both phenoloxidase and quinone methide isomerase. DOPA decarboxylase from *L. cuprina* and from cells of *C. tentans* was inhibited by carbidopa (IC_{50} values of 0·021(±0·011) μM and 0·031(±0·019) μM, respectively) and indomethacine (IC_{50} values of 22·6(±7·1) μM and 18·8(±9·7) μM). Both compounds exerted a competitive type of inhibition and were able to interfere with development of *L. cuprina*.

Key words: *Chironomus tentans*, cuticle, DOPA decarboxylase, insecticide, phenoloxidase, quinone methide isomerase, *Lucilia cuprina*, sclerotization

1 INTRODUCTION

Sclerotization is a key event in cuticle formation of arthropods.[1] During this process enzymatically generated oxidation products of *N*-acetyldopamine and *N*-β-alanyldopamine form covalent cross-links with chitin and cuticular proteins, resulting in chitin–protein and protein–protein complexes which are necessary for hardening and tanning of the cuticle.[2–4] β-Sclerotization[5] and quinone tanning[6] have been identified as pathways leading to sclerotization.[7] Key enzymes of this pathway are phenoloxidases,[3] quinone methide isomerase[4] and DOPA-decarboxylase[3,8] which generate dopamine as the precursor for cross-linking agents and sclerotization. Whereas DOPA- and dopamine-quinone rapidly undergo cyclization and subsequent polymerization to melanin,[9] other *o*-diphenols

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also react with external nucleophilic groups of proteins.[4,10] Quinones, which themselves may have adverse effects on insect tissues, are rapidly inactivated by quinone methide isomerase.[9] This enzyme prevents such deleterious effects by the formation of unstable quinone methides which undergo rapid hydration to non-toxic metabolites.[11–13] Since quinone methides also play a role during encapsulation of foreign matter for self-protection in insects, the related enzymes, together with the key enzymes of the sclerotization pathway, might be suitable targets for interference with arthropod-specific metabolic pathways. A particular target is DOPA decarboxylase which also mediates neurotransmitter generation in nerve tissue.

In this study, the influence of some known inhibitors and several newly synthesized compounds on enzymes involved in sclerotization and tanning was tested in homogenates from the Australian sheep blowfly *Lucilia cuprina* Wied., an economically important ectoparasite. Since determination of DOPA decarboxylase activity is impaired by the simultaneous presence of phenoloxidases in *L. cuprina* homogenates due to oxidation of the substrate, an epithelial cell line of non-biting midges *Chironomus tentans* Walk., which is devoid of this enzyme, was used in this case for inhibitor studies. The results obtained were compared with the effects observed *in vivo*.

2 EXPERIMENTAL METHODS

2.1 Rearing and testing of *Lucilia cuprina* and *Chironomus tentans* cells

Four-day-old pupae of *L. cuprina* were kindly supplied by Dr Stendel, Bayer AG, Wuppertal. Larvae from synchronized cultures (±5 h) were selected at the prepupal stage (120 h) and kept in plastic dishes for a further 96 h at 20–22°C in an 8:16 h light:dark cycle at 75% RH. They were staged according to Filshie.[14] Tests with the larval stages of *L. cuprina* were conducted as follows: to minced horse meat (20 g) in a plastic beaker (5·5 × 4·5 cm) was added the test substance in dimethylsulfoxide (DMSO) + water (2 ml) and 20–50 larvae (first larval stage) were then placed on the meat. The meat-containing beaker was then placed in the bottom of another beaker (9 × 8 cm) containing a 1-cm layer of sterilized sea sand and closed at the top with a perforated Petri dish. The system was then incubated at 26°C and 75% RH. Beakers were checked at fixed intervals for development of the larvae into flies. When all the larvae in the controls had pupated, the meat beakers were removed, the sand sieved and the pupae counted. The pupae were then incubated under the same conditions for another week to determine the number of emerging flies, which were weighed and the weights compared with those in the controls. Tests were performed with pupae of an organophosphate-resistant *L. cuprina* strain (Goondiwindi, Australia). The flies were challenged with a trichlorfon treatment (0.3 mg ml^{-1}) in a six-month rhythm in order to keep the level of resistance high. Significant reversion (80% mortality) of insensitivity after addition of synergists (piperonyl butoxide, 1.5 mg ml^{-1} and tributylphosphorotrithioate 1.5 mg ml^{-1}) indicated that cytochrome P-450-related oxidative metabolism and hydrolases contributed to the level of resistance.

The epithelial cell line from *C. tentans* was obtained from Prof. Dr Lezzi, ETH Zürich, Switzerland and cultured as described by Wyss.[15]

2.2 Sample preparation for enzyme assays

Ten pupae of *L. cuprina* or cells from *C. tentans* culture (35 ml) were homogenized in sodium phosphate buffer (0·1 M; pH 7·0; 5 ml) using an all-glass homogenizer at 0°C. The homogenate was centrifuged (15 000 rev min^{-1}; 17 000*g*) for 30 min at 2°C (Variofuge K., Heraeus, Hanau). With *L. cuprina*, the supernatant was filtered through ice-cold glass wool to remove floating lipids and debris and applied to a Sephadex G 25 superfine column (2·5 × 25 cm) equilibrated with the same buffer. The column was eluted with this buffer (100 ml h^{-1}) and fractions (7 ml) were collected and tested for enzyme activity. 'Active' fractions were pooled and used for kinetic studies and inhibitor testing. Crude homogenates of *C. tentans* cells were used without further purification since the lipid concentration was very low.

2.3 Protein determination

Protein content was determined acording to Bradford[16] using bovine serum albumin as a standard.

2.4 Enzyme assays

Phenoloxidase activity was measured according to Ishaaya.[17] Due to different colours of the test compounds, the optimal absorption range for the photometric assay was determined for each individual reaction product. DOPA decarboxylase assays were performed according to McCaman *et al.*,[18] using [^{14}C]-labelled 3,4-dihydroxyphenylalanine (L-DOPA) as substrate. Quinone methide isomerase activity was tested as described previously,[12] with slight modifications. The reaction mixture (2·9 ml) containing mushroom-tyrosinase (Sigma; 15 U ml^{-1}) and *N*-acetyldopamine (NADA; 1 mM) in sodium phosphate buffer (0·1 M; pH 6·0) was incubated (3 min) until NADA quinone formation had occurred. The biological sample (100 µl)

was then added and isomerase activity was then determined by measuring the decrease in absorbance at 410 nm using an LKB Spectrophotometer Ultrospec. K.

2.5 Test compounds

Compounds I–V (Table 1) were provided by Y. Niederstein and C. Krösche, University of Bonn.[19,20] Inhibitors were dissolved in DMSO and added to the reaction mixture prior to addition of the enzyme preparation. The concentration of DMSO in the test solution did not exceed 3 ml litre^{-1} and control tests revealed no solvent effect on enzyme activities.

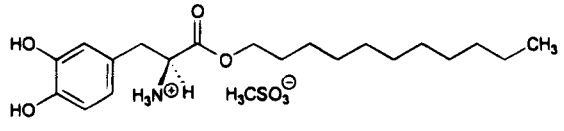
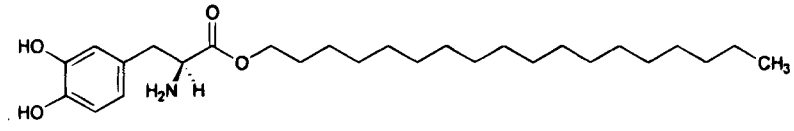
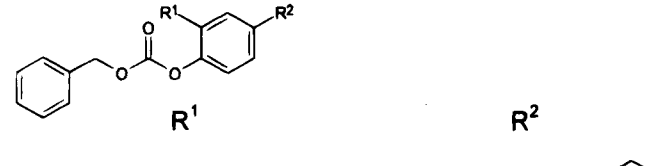
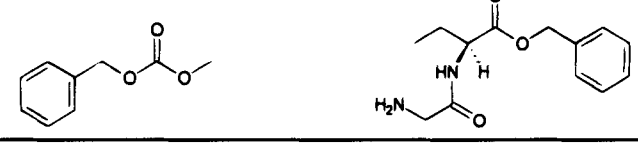
2.6 Statistical analysis

K_m values were computed by transformation of the data according to Lineweaver and Burk.[21] For determination of IC_{50} values, a logit–log transformation was performed. The data were fitted to straight lines by the linear least squares method and IC_{50} values were calculated using Sigma Plot (Jandel Scientific).

3 RESULTS

The activities of quinone methide isomerase, phenoloxidase and DOPA decarboxylase were examined during the development of *L. cuprina* to determine the time period with the highest titer of enzymatic activity which occurred during the early pupal stages with all enzymes (Table 2). DOPA decarboxylase was present in the *C. tentans* cell line without any pretreatment of the cells with moulting hormones, while phenoloxidase activity could not be detected in these cells. Basic characteristics of phenoloxidase and DOPA decarboxylase from *L. cuprina* are presented in Fig. 1. The K_m values for DOPA decarboxylase and for phenoloxidase were 0·47(±0·21) mM and 0·71(±0·16) mM, respectively,

TABLE 1
Inhibition of Phenoloxidase and Quinone Methide Isomerase by Various Derivatives of L-DOPA and L-Tyrosine

	Compound	*Inhibition at 100 μM (%) (±S.D.)*	
		Phenoloxidase[a]	*Quinone methide isomerase*[b]
I		35 (±15)	25 (±11)
II		28 (±9)	100 (±13)
III		14 (±6)	30 (±11)
IV		77 (±13)	65 (±15)
V		69 (±7)	n.i.[c]

[a] 100% corresponds to: Δ 0·042 at 410 nm min^{-1} mg^{-1} protein.
[b] 100% corresponds to: Δ 0·011 at 410 nm min^{-1} mg^{-1} protein.
[c] n.i. = no inhibition.

TABLE 2
Relative Specific Activities of DOPA Decarboxylase, Phenoloxidase and Quinone Methide Isomerase during the Development of *Lucilia cuprina*[a]

Stage of development	*DOPA decarboxylase*	*Phenoloxidase*	*Quinone methide isomerase*
Larvae (L1) 24–36 h	100[b] (±7)	100[c] (±15)	100[d] (±11)
Larvae (L2) 48–60 h	76 (±11)	112 (±15)	81 (±13)
Larvae (L3) 96–120 h	160 (±13)	152 (±17)	41 (±18)
Pupae day 7	496 (±42)	754 (±110)	1101 (±211)
Pupae day 10	91 (±16)	147 (±30)	913 (±163)
Adults	197 (±21)	94 (±14)	195 (±51)

[a] The activities at the larvae L1 stage were set as 100% ($n = 3$; % activity ± S.D.).
[b] 100% corresponds to 0·06 μmol dopamine h^{-1} mg^{-1} protein.
[c] 100% corresponds to Δ 0·0369 at 410 nm min^{-1} mg^{-1} protein.
[d] 100% corresponds to Δ 0·0038 at 410 nm min^{-1} mg^{-1} protein.

using L-DOPA as substrate and that for DOPA decarboxylase from *C. tentans* cells was 0·42(±0·18) mM. Various phenolic compounds could be used as substrates or as effectors for phenoloxidase from *L. cuprina*, the most efficient being norepinephrine, NADA, dopamine and L-DOPA (Table 3). A similar investigation of substrate specificity of quinone methide isomerase revealed that dopamine quinone is formed by mushroom tyrosinase with dopamine as prefered substrate (Table 4). In order to identify new lead structures for anti-sclerotization agents, several derivatives of the key precursors L-DOPA and L-tyrosine were synthesized. Compounds **I** and **II** (Table 1) contained fatty acid residues to improve their transport and/or incorporation behaviour into membrane compartments and were also able to form indoles. These properties might be useful to facilitate 'diffusion' to the epidermis cells and could enhance the inhibitory potential of these substances. Compounds **III**, **IV** and **V** (Table 1) as protected more-lipophilic amino acid mimetics, were intended as competitive inhibitors with enhanced capability to penetrate cellular barriers. None of these compounds affected DOPA decarboxylase, but **IV** and **V** in particular inhibited phenoloxidase at 100 μM, whereas the effect on quinone methide isomerase varied according to the type of derivatization (Table 1). Compound **IV** was the only one which inhibited both enzymes to a similar degree. Among the compounds tested as DOPA decarboxylase inhibitors, carbidopa and indomethacine were by far the most potent inhibitors, not only in *L. cuprina* (Table 5) but also in *C. tentans* cells (Fig. 2).

TABLE 3
Formation of Quinones from Different Catechols by Extracts from *Lucilia cuprina* Pupae at an Early Stage of Development

Catechol	*Relative affinities*[a] (K_m) (±S.D.)
NADA	1 (±0·1)
Dopamine	1·5 (±0·3)
1,2 Benzenediol	75·3 (±3·1)
N-β-Alanyldopamine	0·9 (±0·2)
L-DOPA	0·7 (±0·3)
Epinephrine	3 (±0·5)
Norepinephrine	0·4 (±0·1)
3-methoxy-L-tyrosine	7 (±1·1)
DL-methyl-DOPA	2·3 (±0·3)
Pyrogallol	6·3 (±0·6)

[a] NADA as substrate for phenoloxidase was taken as reference ($n = 3$; K_m = 1·08(±0·23) mM). Means from three determinations with standard deviations are given.

TABLE 4
Quinone Methide Isomerase Activity in Extracts from *Lucilia cuprina* Pupae at an Early Stage of Development

Substrate formed by mushroom tyrosinase from[b]	*Relative activities*[a] (*Factor* ± *S.D.*)
NADA	1 (±0·13)
Dopamine	0·13 (±0·03)
Epinephrine	2·01 (±0·41)
Norepinephrine	0·77 (±0·20)
L-DOPA	1·46 (±0·37)

[a] NADA as substrate was taken as reference (enzyme activity corresponds to Δ 0·0042 at 410 nm min^{-1} mg^{-1} protein). Factors lower than 1 indicate that less substrate is needed to achieve Δ 410 nm values comparable to NADA.
[b] Quinones as substrates for quinone methide isomerase were formed from their corresponding catechols by mushroom tyrosinase.

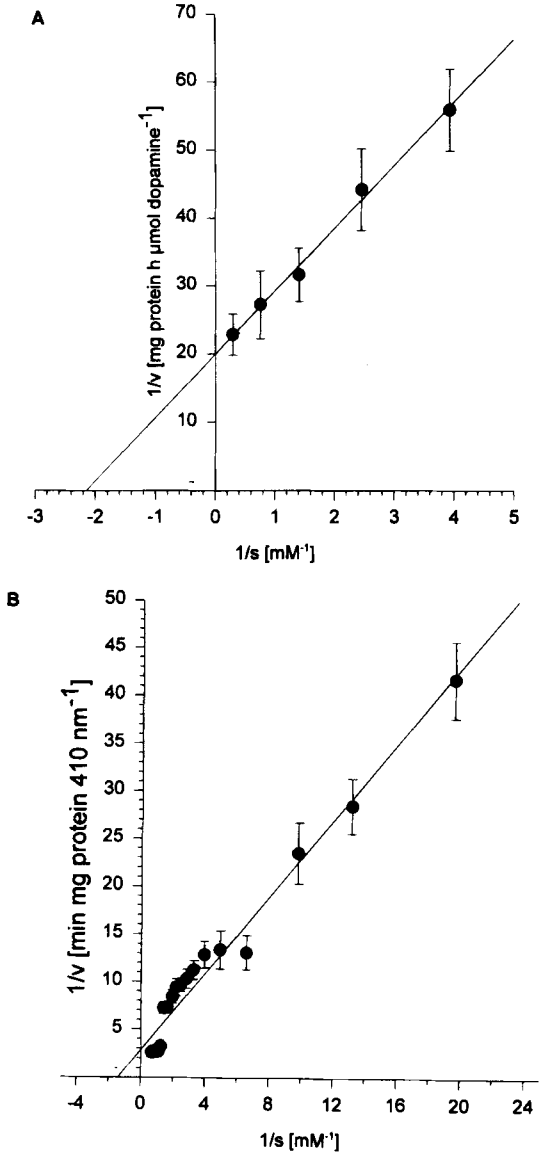


Fig. 1. Determination of kinetic properties of (A) DOPA decarboxylase and (B) phenoloxidase from *Lucilia cuprina* pupae using DOPA as substrate. The data are expressed as Lineweaver–Burk plot. K_m and V_{max} values were 0·47(±0·21) mM and 0·05 µmol dopamine mg^{-1} protein h^{-1} for DOPA decarboxylase and 0·71(±0·16) mM and 0·33 Δ 410 nm mg^{-1} protein min^{-1}.

TABLE 5
Half-maximal Inhibition of DOPA Decarboxylase from *Lucilia cuprina* Pupae at an Early Stage of Development

Compound	*Half-maximal inhibition µM (±S.D.)*[a]
NADA	900 (±113)
L-DOPA	400 (±101)
DL-methyl-DOPA	575 (±162)
Indomethacine	22·6 (±7·1)
Carbidopa	0·021 (±0·011)

[a] Mean of three determinations.

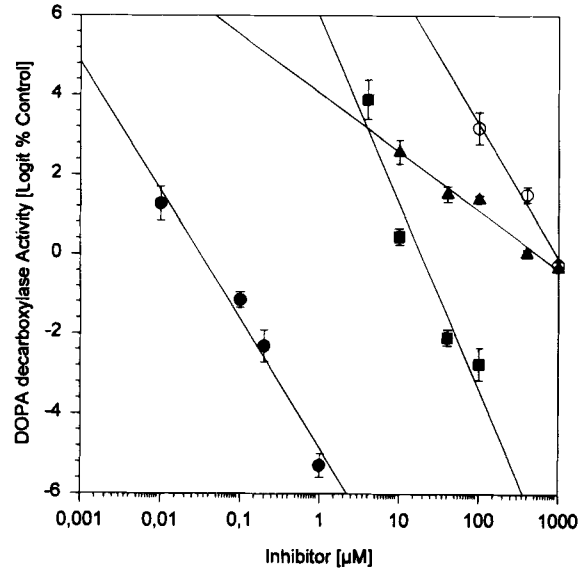


Fig. 2. Influence of (●) carbidopa, (■) indomethacine, (▲) DL-methyl-DOPA and (○) *N*-acetyldopamine on DOPA decarboxylase from *Chironomus tentans* cells. Control value corresponds to 2·7 nmol dopamine mg^{-1} protein h^{-1}. The initial DOPA concentration was 10 µM.

Various compounds displaying structural similarities with the natural substrates of enzymes involved in the sclerotization process were tested for their inhibitory potential. Weak inhibitors of *L. cuprina* DOPA decarboxylase were pyrogallol, 2,5 di-*tert*-butyl-4-hydroxybenzaldehyde, 1,2-benzenediol (IC_{50} = 300, 350 and 400 µM, respectively) and melanin (IC_{50} = 60 µg ml^{-1}), which also slightly inhibited DOPA decarboxylase from *C. tentans* (IC_{50} = 400 µg ml^{-1}). Other potential inhibitors such as thymol, tyramine, tyrosylglycine, hydroquinone, 4-acetamidophenol, 4-aminophenol, 2,6-di-*tert*-butyl-4-methylphenol, 2,6-di-*tert*-butylphenol, 3-hydroxy-D,L-kynurenine, 4-methylphenol, 3-nitrotyrosine, D,L-octopamine, phloroglucinol, plumbagin and resorcinol did not inhibit either DOPA decarboxylase, phenoloxidase or quinone methide isomerase or showed very poor inhibition. The IC_{50} values for these substances were all >1 mM.

There was a considerable loss in the concentration of L-DOPA due to the oxidative activity of tanning enzymes, especially phenoloxidases. This led to variable and less accurate values for DOPA decarboxylase activity and addition of ascorbic acid (5 mM) as an antioxidant[22] only partially improved this situation. Therefore, inhibitor studies with DOPA decarboxylase were performed with homogenates from the epithelial cell line from *C. tentans*, which is devoid of phenoloxidases and displayed similar characteristics to those of the *L. cuprina* enzyme. As shown in Fig. 2, carbidopa was the most effective inhibitor, displaying an IC_{50} value of 0·03(±0·021) µM, followed by indomethacine with a value of 18·8(±13·7) µM while D,L-methyl-DOPA (559(±182) µM) and NADA (945(±281) µM) were much

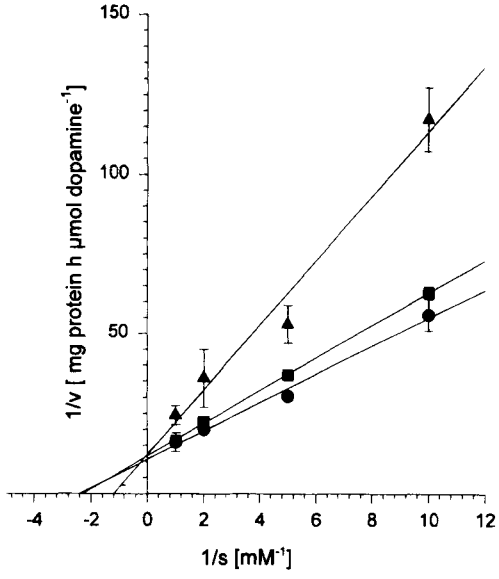


Fig. 3. Inhibition of DOPA decarboxylase from *Chironomus tentans* cells by carbidopa. The data are expressed as Lineweaver–Burk plot. (▲) 3 µM carbidopa, (■) 0·3 µM carbidopa, (●) without inhibitor.

less active. Both carbidopa (Fig. 3) and indomethacine, (data not shown) revealed a competitive type of inhibition.

The most effective enzyme inhibitors, carbidopa, indomethacine (DOPA decarboxylase) and compounds **II**, **IV** and **V** (phenoloxidase and/or quinone methide isomerase) were tested for their activity against larvae. As shown in Table 6, with the exception of **II**, the compounds revealed some activity against *L. cuprina*. Whereas carbidopa directly killed all larvae at the next moulting stage, indomethacine and compounds **IV** and **V** caused 40–70% inhibition of *L. cuprina* development at the early pupal stage, when a dose of 0·5 mg compound g^{-1} food was applied. In addition, about 50% of the pupae had a reduced weight compared with the control and a decrease in the number of the surviving adults was also observed.

TABLE 6
Activity of DOPA Decarboxylase, Phenoloxidase and Quinone Methide Isomerase Inhibitors against *Lucilia cuprina* Larvae

Compound	*Mortality*[a] *at 0·5 mg g^{-1}* (%) (±*S.D.*)	*Stage affected*[b]
Carbidopa	100	2nd larvae
Indomethacine	71 (±13)	Early pupae
II	0	None
IV	40 (±10)	Early pupae
V	69 (±8)	Early pupae

[a] Compounds were dissolved and added to the larval food at the beginning of the test.
[b] Larvae were monitored daily for activity at each stage of development until hatching of the adults occured.

4 DISCUSSION

The sharp rise in DOPA decarboxylase and phenoloxidase activity at the beginning of puparium formation is in accordance with the onset of the tanning process of the cuticle during this developmental stage and is also observed in a variety of other insects. Similar titer profiles of phenoloxidases have been observed during development of the Egyptian cotton leafworm *Spodoptera littoralis* Boisd.,[17] the citrus bud mite *Aceria sheldoni* Ewing[23] and the Californian red scale *Aonidiella aurantii* Maskell[24] and for DOPA decarboxylases from *L. cuprina*,[25] the Madeira cockroach *Leucophaea maderae* (F.),[26] the American cockroach *Periplaneta americana* L.,[27] the blowfly *Calliphora vicina* Robineau-Desvoidy[28] and the vinegar fly *Drosophila melanogaster* Meig.[29] The Michaelis–Menten constants for DOPA decarboxylases from *L. cuprina* and *C. tentans* cells respectively, as well as for *L. cuprina* phenoloxidase correlate with comparable data for DOPA decarboxylase from *C. vicina*[28] and cuticular phenoloxidase from the silkworm *Bombyx mori* L.[30] Several compounds were tested as substrates for *L. cuprina* phenoloxidases. The *L. cuprina* enzyme accepted *o*-diphenols, known to be key substrates for sclerotization, such as L-DOPA, dopamine and *N*-β-alanyldopamine (NBAD) much more readily than monophenols such as tyrosine and tyramine. Similar observations regarding substrate specificity were reported for enzymes from *B. mori*[30] and the rice moth *Corcyra cephalonica* Stainton.[31] The *o*-diphenoloxidase described for *L. cuprina* resembles the cuticular-bound phenoloxidase from *B. mori* and the enzyme from *C. cephalonica* with respect to substrate specificity and to the pattern of expression during development, indicating that this type of enzyme is important for quinone tanning and melanin formation in pupal cuticle.[30,31] The substrate specificity of quinone methide isomerase roughly parallels that of the prefered phenoloxidase substrates. When synthesized by mushroom tyrosinase, dopamine quinone, norepinephrine quinone and *N*-acetyldopamine quinone were used preferentially compared with epinephrine and L-DOPA quinone. This is in accordance with the assumed function of the enzyme in quinone methide sclerotization.[9]

So far, no selective inhibitors of cuticle sclerotization are known. Possible candidates could be antioxidants or inhibitors of quinone methide isomerase which interfere with the benzylic deprotonation of *N*-acetyldopamine quinone in a highly stereoselective reaction.[32] We have previously synthesized various derivatives and analogues of *N*-acetyldopamine and

found that *N*-acetyl-2,4-dihydroxyphenethylamine inhibits the incorporation of NBAD into the cuticle of the tobacco hornworm *Manduca sexta* (Joh.) *in vitro* during the tanning process.[33] This was explained by the trapping of quinonoid intermediates of catechols by the resorcine derivative.[34] However, none of those research concepts to identify new insecticides has led to compounds that are useful for practical applications. Based on the assumption that severe disturbance of sclerotization might be easier to achieve if more than one of the key enzymes were significantly inhibited by the same compound, we were particularly interested in compounds which inhibit phenoloxidase and quinone methide isomerase simultaneously. This effect was most pronounced in compound **IV**. Whereas **II** revealed a nearly complete inhibition of quinone methide isomerase, **V** impeded only phenoloxidase activity. Thus, a test to compare these new inhibitors with DOPA decarboxylase inhibitors was performed, in order to evaluate the significance of specific enzyme inhibition for growth inhibition *in vivo* and mortality.

Although the inhibitors of phenoloxidase and quinone methide isomerase displayed only weak effects *in vivo* against *L. cuprina*, the rise in enzyme activity during larval development at the early pupal stage indicates that these enzyme targets play a crucial role, particularly during pupal development. The inhibition *in vitro* of phenoloxidase by the long-chain alkyl derivatives of L-DOPA, i.e. **I** and **II**, could be due, at least partly to a rather unspecific detergent effect of these compounds which could be observed even at low concentrations. Since relatively large amounts of NADA and NBAD presumably accumulate in the cuticle before pupation, incomplete inhibition of phenoloxidase and/or quinone methide isomerase might not lead to a deficiency in tanning compounds sufficient to suppress sclerotization. This could also be a reason for the delayed effect of indomethacine, whereas the level of inhibition by carbidopa *in vivo* indicates that sufficient enzyme inhibition also occurs under these conditions. From these studies it can be concluded that inhibitors of DOPA decarboxylase might be particularly useful as target compounds. DOPA decarboxylase is also involved in the mammalian pathway for catecholamine synthesis, so that enzyme inhibitors may not appear to be highly selective for parasites. Indeed, it was shown that the enzyme from rat brain was just as effectively inhibited by various inhibitors as DOPA decarboxylase from *L. cuprina*.[25] However, the inhibitors seem to be much less toxic to mammals than, for example, to blowfly larvae,[25] which might be a reflection of the fact that compounds such as carbidopa are unable to penetrate the blood–brain barrier.[35]

DOPA decarboxylase is not only expressed in epidermal tissues but is also present in neurons and hemolymph blood cells of insects.[26,36] Thus, an inhibitor of this enzyme might also lead to neuronal disturbances. Indeed, the relatively early and fast action of carbidopa on *L. cuprina* larvae might be related to neuronal effects to some extent. However activity *in vivo* could also be drastically influenced by pharmacokinetic and metabolic effects besides enzyme inhibition at the target site.

The inhibition of DOPA decarboxylase by various compounds has already been described as an interesting target site for interference with insect sclerotization and growth.[25] In this context, carbidopa and D,L-α-methyl DOPA were shown to inhibit the enzyme from *L. cuprina* effectively and they also displayed activity against larvae.[25] For D,L-α-methyl-DOPA-treated larvae, ultrastructural investigations of cuticle revealed a severe disturbance of the fine structure and permeability properties leading to larval death, most likely due to rapid dehydration.[37] The good activity shown *in vitro* and *in vivo* by carbidopa is in contrast to observations by Turnbull and Howells[25] who described an apparent K_i value for the most active D,L-α-methyl-DOPA in the μM range and LD_{50} values for first- and second-instar larvae ranging from 1·2 to 1·8 × 10^{-4} M. The weak activity of D,L-α-methyl-DOPA against our *L. cuprina* strain could be due to inactivation within insect tissues caused by enhanced metabolic degradation in this organophosphate-resistant strain.

Indomethacine was found to be a potent inhibitor of *L. cuprina* DOPA decarboxylase *in vitro*, but the compound displayed only weak activity *in vivo*. This compound belongs to a group of non-steroidal, anti-inflammatory amino acid decarboxylase inhibitors and inhibits five different amino acid decarboxylases from rats.[38] DOPA decarboxylase was not investigated in that study. The weak activity *in vivo* might also be explained by degradation and failure of the substance to penetrate the larval cuticle successfully and thus to reach its enzyme target site within epidermal cells. The modes of inhibition of carbidopa and indomethacine were further investigated using enzyme preparations from *C. tentans* cells, since this epithelial cell line does not express DOPA-oxidizing enzymes and be assumed as a source exclusively for cuticular DOPA decarboxylase because it contains no neuronal cells. Both carbidopa and indomethacine competitively inhibited DOPA decarboxylase. For indomethacine this is in accordance with results obtained for different analogues of tryptophan against hog kidney DOPA decarboxylase, where a competitive type of inhibition was demonstrated.[39] It is also well-known that hydrazine-containing carbonyl trapping agents, which are able to form Schiff's bases and can react with pyridoxal and/or pyridoxal-phosphate, inhibit decarboxylation.[40] Similar effects might also be involved during inhibition by carbidopa.

So far, none of the inhibitors investigated has displayed sufficient biological activity to justify commercial development. However, DOPA decarboxylase in particular, and to some extend phenoloxidase and quinone

methide isomerase, have been demonstrated to be promising target sites for larvicide attack. The comparison of the results of the enzyme tests gained *in vitro* with the observed effects *in vivo* indicates that the data obtained by enzymatic tests enable the inhibitory potential of compounds in investigated pests to be predicted. In contrast to the determination of LD_{50} values, in-vitro test systems also give insights into the mode of action of pesticide candidates and can guide directed drug design.

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